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Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA

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Abstract

Yellow head virus (YHV) is an invertebrate nidovirus which causes a severe mortality in cultured *Penaeus monodon*. The mortality may be prevented by prior treatment of shrimps with YHV-protease dsRNA. Whether the YHV infected shrimp might be cured by the dsRNA remains to be investigated. *P. monodon* injected with 10^{-6} YHV showed a high virus replication and mortality within 2 days. Injection of 25 μg YHV-protease dsRNA at 3, 6, 12 or 24 h post YHV infection showed a strong inhibition of YHV replication up to 12 h. Unrelated dsRNA-GFP showed no inhibition, indicating that the inhibition was nucleic acid sequence specific through RNAi pathway. Shrimp mortality could be prevented at 3 h post YHV infection by the dsRNA, but not at 24 h. These results demonstrate that YHV-protease dsRNA gives therapeutic effect and pave the way to develop a cure for YHV-infected shrimps.

Keywords: Yellow head virus (YHV); RNAi; antiviral; dsRNA-protease

1. Introduction

Yellow head disease is a fatal infection in penaeid shrimp caused by yellow head virus (YHV), an invertebrate virus with positive sense single-stranded RNA genome classified as nidovirus (Cowley and Walker, 2002; Sittidilokratna et al., 2002). YHV has caused rapid mass mortality of farmed penaeid shrimp and remains a serious problem to shrimp production industry worldwide. In Thailand alone, estimated loss in shrimp production was about 1 billion U.S.$ between years 1997 and 2000 (Flegel, 2006). Currently, there is no effective drug or vaccine to treat or prevent yellow head disease. Development of new strategy to control YHV therefore represents a great challenge in shrimp aquaculture industry.

RNA interference (RNAi) is a process by which double-stranded RNA (dsRNA) yielding siRNA induces degradation of homologous messenger RNA (mRNA) in a sequence specific mode at post-transcriptional level (Fire, 1999; Tuschl et al., 1999). Accumulated researches from diverse fields described RNAi as a mechanism in which eukaryotes knock down expression of specific genes (Hannon, 2002). This RNA-associated mechanism allows cells to control the expression of undesirable mRNA of either exogenous or endogenous origin. Based upon this scenario, RNAi is therefore considered as a primitive form of natural defense mechanism for eliminating the intruding foreign RNA including virus (Lu et al., 2005).

Recent studies demonstrated that introduction of dsRNA into shrimp prior to viral challenge can prevent viral propagation and shrimp mortality (Robalino et al., 2004, 2005; Yodmuang et al., 2006). It is not known if dsRNA given post-viral infection is still effective. In this study, we aim to investigate whether the dsRNA-triggered RNAi is functional in a curative mode in the shrimp.

2. Materials and methods

2.1. Shrimp rearing

Ten to 12 g healthy *Penaeus monodon* juveniles were used in all experiments. Shrimps were reared in 80 l tank filled with 40 l aerated artificial seawater at 10 ppt salinity and fed with commercial diet. Water was changed every 2 days.

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2.2. Virus stock

YHV was infected into 200 juvenile *P. monodon* shrimps with average weight ~15 g by intramuscular injection. Forty-eight hours post-infection, hemolymph from the shrimps was collected and pooled. YHV in the hemolymph was purified using Urograffin (Schering) gradient centrifugation (Assavalapsakul et al., 2005). The purified YHV was aliquot and stored frozen at −80 °C until use.

To titer the viral stock, primary culture of lymphoid (Oka) organ cells was prepared and grown to 100% confluent in 96-well plate as previously described (Assavalapsakul et al., 2005). Serial 10-fold dilutions of purified YHV were prepared in the same culture medium then inoculated into four wells of the primary cells and incubated for 90 min at room temperature. After incubation, the virus was discarded and fresh medium was added. The cells were grown at 26 °C for 7 days. The virus infectivity was identified by crystal violet staining. Viral titer was determined by $TCID_{50}$ end points as described by Reed and Muench (1938). The viral stock used in this study was ~3×10^9 infectious virions.

2.3. dsRNA preparation

dsRNA-protease and dsRNA-GFP was prepared by over-expression in *Escherichia coli* HT115 as previously described (Ongvarrasopone et al., in press). *E. coli* HT115 containing the recombinant plasmid with inverted sequence of YHV protease or of GFP was inoculated into 2 × YT medium and cultured until OD_{600} of 0.4 at 37 °C. The expression of hairpin RNA of the corresponding gene was induced by addition of IPTG to final concentration of 0.4 mM for 4 h. One OD_{600} ml of bacterial cell was pelleted by centrifugation and resuspended in 50 μl phosphate saline buffer (PBS) containing 0.1% SDS. The sample was boiled for 2 min then snapped cool on ice. Single-stranded RNA in the loop region of hairpin structure and endogenous RNA from the bacterial host strain was eliminated by addition of 1 μg RNAse A in RNase A buffer (300 mM sodium acetate, 10 mM Tris–HCl, pH 8.0) and incubated for 15 min at 37 °C. Double stranded RNA was extracted from bacterial lysate by TRI reagent (Molecular Research Center) according to manufacturer protocol. Each dsRNA obtained from this preparation appears as a single band in agarose gel electrophoresis corresponded to its expected size. The dsRNA integrity was confirmed by RNase III and RNase A digestion. DsRNA concentration was estimated by OD_{260} and adjusted to final concentration of 1 μg/μl prior to storage at −80 °C until use.

2.4. Shrimp injection and sample processing

For YHV inhibition assay, shrimps were challenged with YHV 10^{-6} dilution in PBS (approximately 3×10^3 virions), by injecting 50 μl into hemolymph using 1 ml syringe with 29 gauge needle. Such injection usually resulted in total mortality within 2–3 days. The infected shrimps were then injected with 25 μg dsRNA-protease, dsRNA-GFP or 150 mM NaCl into the hemolymph at indicated time post-infection. To determine viral load, hemolymph was collected 48 h after the viral challenge for RNA extraction and RT-PCR analysis. Gill sample from the same shrimp was collected for YHV antigen determination by Western blot analysis.

For mortality assay, healthy *P. monodon* juveniles (~10 g) were used (10 shrimps/group). Same procedure for YHV inoculation and dsRNA injection was performed as described above. The mortality was recorded twice a day for 10 days after YHV challenge. Statistical analysis of the mortality test was performed using Fisher's exact test.

2.5. RT-PCR analysis

Total RNA was isolated from 200 μl of hemolymph from individual shrimps using TRI-LS reagent (Molecular Research Center). First strand cDNA was prepared from 1 μg of total RNA using oligo-dT and Improm pt II reverse transcriptase (Promega) as described by manufacturer. The level of YHV in each RNA sample was monitored by multiplex PCR. Primer pairs specific to YHV helicase gene (5′-CAA GGA CCA CCT GGT ACC GGT AAG AC-3′ and 5′-GCG GAA ACG ACT GAC GGC TAC ATT CAC-3′) was used to monitoring YHV level whereas primer pairs for *P. monodon* actin (5′-GAC TCG TAC GTG GGC GAC GAG G-3′and 5′-AGC AGC GGT GGT CAT CTC CTG CTC-3′) are for internal control. The PCR amplification was performed for 30 cycles and the PCR product was analyzed by 1% agarose gel electrophoresis.

2.6. Western blot analysis

Detection of YHV antigen was performed similar to that previously described (Yodmuang et al., 2006). Equal amounts of protein (~50 μg) prepared from gills of individual shrimp in 2× sample buffer were resolved in sodium dodecyl sulfate polyacrylamide gel (8%) electrophoresis prior to transferring to PVDF membrane (BioRad). The membrane was blocked with 5% skim milk in PBS containing 0.2% Tween-20 for 1 h at room temperature. The membrane was probed with mouse antiserum raised against gp116 of YHV (dilution 1:2000) for 1 h and followed with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibodies (Sigma Chemical) (dilution 1:8000). The gp116 was detected using the ECL Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech).

3. Results

3.1. dsRNA exerts therapeutic property for YHV infection in shrimp

Previously, we have shown that pre-administration of *P. monodon* with dsRNA-protease 24 h prior to YHV inoculation efficiently prevented the viral propagation and protected the shrimp from mortality (Yodmuang et al., 2006). To investigate whether the dsRNA mediated YHV inhibition is capable of inhibiting the virus in shrimps that were already infected. YHV at 10^{-6} dilution was injected into hemolymph of *P. monodon* allowing the virus to multiply for 3 h. The shrimps were then